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Detailed protocol

RNA interference (RNAi) protocol for down regulation of cytochrome *CYP306A1* expression in the main malaria vector *Anopheles gambiae*.

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[Abstract]

In sub Saharan Africa, mosquitoes of medical importance include *Anopheles gambiae*, one of the main malaria vectors. This and other vectors are predominantly controlled through the use of chemical insecticides. However, new targets for vector control are urgently needed to reduce malaria transmission due to the spreading of the insecticide resistance phenomenon. In *Anopheles gambiae*, a likely candidate for genetic control is the steroid hormone 20-hydroxyecdysone (20E). Target disruption of this hormone synthesis pathway could lead to the identification of novel chemical or genetic targets for the implementation of alternative vector control interventions. We developed an RNA interference protocol targeting the cytochrome P450s *CYP306A1* involved in the early biosynthesis of the 20E in *An. gambiae* mosquitoes. Here we describe the design and the methodology of this RNAi technique. This interesting tool is broadly applicable to any gene of interest and could be expanded to other malaria-transmitting vector species.

Keywords: *Anopheles gambiae*, 20-hydroxyecdysone, *CYP306A1*, RNA interference, malaria.

[Background]

In the tropical countries worldwide, malaria is one of the vector borne diseases that remains a major public health concern, especially in sub-Saharan Africa (Ogola et al., 2018). This diseases is transmitting through the bite of *Plasmodium* spp. infected female mosquitoes belonging to the genus *Anopheles* (World Health Organization, 2020). Indeed, *Anopheles gambiae* is one of the main malaria vectors in the African region where the highest number of malaria cases and deaths are reported (Tchouassi et al., 2012; World Health Organization, 2021). Among the current malaria control strategies (prevention, diagnostic and treatment), the prevention programs includes the use of chemical insecticides for mosquito populations control contributing significantly to the reduction of malaria incidence (Bhatt et al., 2015). However, spreading of insecticide resistance in natural mosquito populations is threatening the effectiveness of current vector control implementation (Kamgang et al., 2018; Tchouakui et al., 2018). Additional targets for vector control intervention are urgently needed to reduce malaria transmission. New technologies for malaria-transmitting vector control could be achieved either by blocking parasite development or by reducing the reproductive capability of these insect vectors using novel transgenic tools. This can potentially lead to powerful innovative interventions to reduce malaria transmission.

One of the genetic targets in the vector, are genes encoding the cytochromes P450 (CYP) enzymes. These are hemoproteins that play a fundamental role in the metabolism and detoxification of many structurally and chemically diverse compounds (Manikandan & Nagini, 2018; Nair et al., 2016). P450s are monooxygenases present in all insect tissues and are responsible for several functional roles, including growth, development, feeding and protection against xenobiotics, including metabolism of pesticides as well as tolerance to plant toxins (Scott & Wen, 2001). Furthermore, P450 enzymes are involved in the biosynthetic pathways of ecdysteroids and juvenile hormones, which are key chemical agents of insect growth, development, and reproduction (Feyereisen, 1999). The main ecdysteroid in insects, including mosquitoes, are ecdysone and 20-hydroxyecdysone (Lafont et al., 2017).

The *CYP306A1*, a 25-hydrolyase monoxygenase, is involved in the first steps of ecdysone biosynthesis in mosquitoes. Indeed, *CYP306A1* converts 2,22,25-trideoxyecdysone (3b,5b-Ketodiol) into 2,22-dideoxyecdysone (3b,5b-Ketotriol) in the early step of the steroid hormone synthesis (Warren et al., 2006). In *Anopheles gambiae*, the steroid hormone 20E modulates multiple physiological processes (reproductive success, parasite development, and longevity) that impacts on their competence to transmit malaria (Mitchell et al., 2015). Thus, target disruption of the ecdysone and 20E biosynthesis could negatively impact mosquito life-history traits and consequently affect the mosquito vectorial capacity. The present aims at down regulating the gene expression of *CYP306A1* using the RNA interference (RNAi) assay in *Anopheles gambiae* female mosquitoes. Here we describe in details the design, the step-by-step methodology and the limitation of this silencing technique.

Materials and Reagents

1. Double-distilled or Milli-Q water
2. Hard-Shell® 96-Well PCR Plates (Bio-Rad, catalog number: HSP9655)
3. 0.2 mL PCR Tubes (Thermo Scientific™, catalog number: AB0620)
4. Primers qPCR (Inqaba)
5. Primers T7 (Macrogen)
6. Ethanol absolute (VWR Chemicals, AnalaR NORMAPUR, catalog number:20821.330)
7. Sodium acetate (Merck Chemicals LTD, Saarchem, catalog number: AAJ61934AK)
8. Agarose (Lonza, Seaken LE Agarose, Catalog number: 50004), store dry (18-26°C)
9. 3.5" replacement tubes (Drummond Scientific Company, catalog number:3-000-203-G/X)
10. Laboratory counter (BAL Supply LLC, model 202C)
11. Filter paper 3HW (AHLSTROM Munktell, catalog number: 3.303.185)
12. Parafilm (Bemis, model: PM-996)
13. Picopure RNA isolation Kit (Applied Biosystems, cataloge number: KIT0204)
14. RNase-Free DNase Set (Qiagen, cataloge number: 79254)
15. SuperScript III first strand synthesis kit (Invitrogen, cataloge number: 18080051)
16. Brilliant III SYBR Green master mix (Agilent Technologies, cataloge number: 600882)
17. qPCR plate seals (ThermoFisher scientific, cataloge number: AB1170)
18. Takara Taq DNA polymearase (Takara Bio Group, cataloge number: R001AM)
19. QIAquick1 PCR purification kit (Qiagen, cataloge number: 28104)
20. MEGAscript RNAi kit (Invitrogen, cataloge number: AM1626)

Equipment

1. Centrifuge (HERMLE labortechnik GmbH, HERMLE, model: Z 400K)
2. Microcentrifuge (Kendro laboratory, Heraeus, model: BIOFUGE pico)
3. Mini plate spinner (Labnet International, model : C1000-230V)
4. PCR plate sealer (BIO-RAD, model : PX1^{TM} PCR plate sealer)
5. Nanoject II (Drummond Scientific Company, catalog Number: 3-000-205/206)
6. Needle puller (Narishige group, NARISHIGE, model :PC-100 puller)
7. Nanodrop Spectrophotometer (Thermo Scientific, model: Nanodrop 2000c)
8. Micropipettes (Thermo Labsystem, Finnpipette P2, P20, P200, P1000)
9. Dry bath (Labnet International, Accublock Digital Dry bath, model: D1200)
10. DNA electrophoresis system
11. Thermal cycler (BIO-RAD, model :T100^{TM} Thermal cycler)
12. C1000 Touch thermal cycler (BIO-RAD, model: CFX 96^{TM} Optics module)

Software

1. Software 1 name (Company or Developer/Provider/Supplier, web address)
2. Software 2 name (Company or Developer/Provider/Supplier, web address)
3. ImageLab 6.0 for gels visualization (BIO-RAD, www.bio-rad.com)
4. Graph pad prism (San Diego, California, USA)

Procedure

A. **Mosquito colony and rearing**

In the framework of this protocol, we use the *Anopheles gambiae* COGS colony (Koekemoer et al., 2010), maintained in the Botha De Meillon Insectary in Johannesburg. Mosquitoes are reared under standard insectary conditions of 25°C, 80% relative humidity with a 12-h day/night lighting regime and 45-min dusk/dawn cycles (Hunt et al., 2005).

A. **Oligonucleotides**

1. ***qPCR Primers design***

Oligonucleotides for quantitative reverse transcription (qRT-PCR) are designed to evaluate the relative expression of the *CYP306A1* (Vectorbase accession number: AGAP004665) to confirm the silencing. Primers pair to amplify the reference control gene *Rsp7* (Vectorbase accession number: AGAP010592) are also designed. Primers are designed using the PrimerQuest™ Tool available at https://eu.idtdna.com/PrimerQuest/Home/Index. All primers are synthetized by Inqaba biotec (Pretoria, South Africa) with standard purification (**Table 1**).

1. **T7 primer design for dsRNA synthesis**
    a. Select a region of 500 bp of the gene as target for the dsRNA synthesis.
    b. Design sequence-specific primers to the unique dsRNA region.
    c. Tail the primers sequences with T7 Promoter region (*TAATACGACTCACTATAGGAGA*) at 5′ end of the oligonucleotides to allow for *in vitro* transcription to take place.
    d. Design a primer pair to amplify the Green Fluorescent protein (GFP) which is used as control. All T7_primers are synthetized by Macrogen (Geumcheon, Korea) and purified by Macrogen Oligonucleotide Purification Cartridge (MOPC) method (**Table 1**).

Notes: *The dsRNA primers are on a different region of the taget gene transcript so they don't overlap with the qPCR primers.*

Table 1: Sequences of oligonucleotides

Table 1: Sequences of oligonucleotides

	Primers	Sequences 5’ to 3’
qRT-PCR	306A1-F1	GGCAGGTGTGGATACTACATTT
	306A1-R1	CGACCAGTCTTTGTCTCAGTTC
	Rsp7-Fwd	CACCGCCGTGTACGATGCCA
	Rsp7-Rev	ATGGTGGTCTGCTGGTTCTT
T7 primers	*GFP_F*	GAATTGTAATACGACTCACTATAGGGCATGGTGAGCAAGGGCGAG
	GFP_R	GAATTGTAATACGACTCACTATAGGGCTTACTTGTACAGCTCGTC
	306A1_T7 Fwd3	**TAATACGACTCACTATAGGAGA**CTGGTCGTGAATGAGCC
	306A1_T7 Rev1	**TAATACGACTCACTATAGGGAGA**AGTCTGGGGGAGTTAAGGTT

The T7 sequence is attached to the 5’ end of the primers (in bold).

A. **RNA isolation and first DNA strand (cDNA) synthesis**

1. Extract total RNA from 5 days old COGS adult females using PicoPure RNA isolation kit (Qiagen) according to the manufacturer instructions.
2. Treate the isolated RNA sample with RNase-Free DNase Set (Qiagen) according to the manufacturer instructions.
3. Measure RNA concentrations using Nanodrop spectrophotometer (Nanodrop Technologies, Wilmington, DE, USA) and the RNA extracts are stored at -80°C until further use.
4. Perform the first DNA strand (cDNA) synthesis with 2.5 µg of RNA using the SuperScript III first strand synthesis kit (Invitrogen) according to supplie instructions.

A. **qPCR primers validation**

Primer validation is done through standard curve production to determine PCR efficiency and specificity of each primer pair.

1. Proceed to a serial dilution of stock cDNA by making 5 dilutions from the original cDNA stock as depicted in **Figure 1**.

Figure 1: cDNA dilutions preparation

1. Perform the qPCR reactions by analyzing all five dilutions in duplicate plus a negative control for each primer pair. Typical qPCR conditions are (Table 2).
   1. µL

Table 2: Overview of qPCR conditions

Components	20 µL reaction
Brilliant III SYBR Green master mix	10 µL
10 µM forward primer	0.6 µL
10 µM reverse primer	0.6 µL
cDNA dilution	1 µL
Nuclease free water	

1. Load all reaction components into the appropriate wells plate and seal the plate.
2. Spin the plate at 3000 rpm for 1 min.
3. PCR conditions for all primers are 95 °C for 3 min to activate the hot-start polymerase, followed by 40 cycles (95 °C for 10s, gradient of 52°C to 62 °C for 20s), a step of 1 cycle of 72°C for 1 min, and a final step of 55°C to 95°C for 5s (melt curve), with fluorescence measured after each cycle.
4. Evaluate the efficiency (E) and the R^2 coefficient.

A. **PCR using T7 primers**

The specificity of the T7_primers to amply the target region of the gene is evaluated by carrying out PCR reactions. This PCR step will provide double stranded DNA template from the cDNA.

1. Prepare PCR mix as in Table 3 below:
   1. µL

Table 3: Overview of PCR conditions

Components	25 µL reaction

10X Taq Buffer	2.5 µL
dNTP mixture	2 µL
Takara Taq	0.2 µL
Forward T7_primer (10 pmol/µL)	0.25 µL
Reverse T7_primer (10 pmol/µL)	0.25 µL
$MgCl_2$ (25 mM)	1 µL
cDNA	1 µL
Nuclease free water	

1. Perform PCR by heating the sample to 95˚C for 4 minutes, followed by 40 PCR cycles (95˚C for 30 seconds, 55˚C for 20 seconds, 65°C for 55 seconds, 68˚C for 55 seconds), and a final extension at 68˚C for 10 minutes.
    1. Purify the PCR products using the QIAquick PCR purification kit (Qiagen), as per the manufacturer's specifications.

D. PCR products purification

After PCR reactions using the T7_primers:

1. Eluted in 30 µL of elution buffer.
2. Quantify the purified PCR products using the NanoDrop™ spectrophotometer.
3. Examine the integrity and size of the purified PCR products on a 2% agarose gel (30 minutes, 300V, 400 mA, in TAE (Tris/Acetate/EDTA) buffer), prior to *in vitro* transcription.

E. dsRNA synthesis

1. In a 20 µL reaction, add 2 µL of each dNTP (ATP, CTP, GTP, UTP) solution, 2 µL of T7 enzyme mix and 2 µL of 10X T7 reaction buffer to 1 µg dsDNA template.
2. Incubate the samples at 37˚C overnight, following by a subsequent incubation at 75°C for 5 minutes.
3. Allow reactions to cool on the bench at room temperature (Do not put the tube on ice).
4. Run RNA on 2% agarose gel using 5 µL of a 1/100 sample dilution (30 minutes, 300V, 400 mA, in TAE (Tris/Acetate/EDTA) buffer).

Notes: The MEGAscript RNAi kit (Invitrogen) is used for transcription reactions according to the manufacturer's instructions.

A. **Nuclease digestion to remove DNA and ssRNA (single stranded RNA)**

For digestion reactions:

1. Add 5 µL of 10X digestion buffer, 2 µL of DNaseI, 2 µL of RNase, and 21 µL nuclease-free water to the *in vitro* transcribed dsRNA products.
2. Incubate the samples for 1 hour at 37˚C to allow for the template DNA to digest.

Notes: Perform nuclease digestion to digest residual template DNA and ssRNA from the new synthetised dsRNA.

A. **Purification of dsRNA**
    1. Use the filter cartridges supplied in the MEGAscript RNAi kit, as specified by the manufacturer.
    2. Heat the provided elution solution at 95°C.
    3. Elute the purified *in vitro* transcribed dsRNA in 60 µL of heated elution solution
    4. Quantify the dsRNA using the NanoDrop™ spectrophotometer after a 1:10 dilution of dsRNA.
    5. Assess the integrity of dsRNA on a 2% agarose gel, TAE (30 minutes, 300V).

Notes: After digestion, this cleaning step eliminates proteins, free nucleotides, and digested nucleic acid products from the dsRNA samples.

A. **Ethanol precipitation**

1. Measure the volume of dsRNA samples.
2. Add 1/10 volume of sodium acetate (CH_3COONa, pH 5.2) and mix well.
3. Add 2.5 volumes of ice-cold 100% ethanol and mix well.
4. Place at -20°C for 5 hours (or at -80°C for 1 hour).
5. Spin at 13000 rpm for 30 minutes, 4°C.
6. Carefully decant supernatant.
7. Add 1 mL 70% ethanol, finger flic tube and spin briefly at 13000 rpm for 5 minutes, 4°C.
8. Carefully decant supernatant.
9. Air dry pellet on the bench for 2 hours.
10. Suspend pellet in 20 µL of nuclease free water.

Notes: This step is in order to prepare and concentrate dsRNA for injection.

A. **dsRNA delivery**

Here, we use the Nanoinject II (Drummond, 3–000204) to conduct dsRNA inoculation on 2-days old female *Anopheles gambiae* mosquitoes (COGS colony) following the described method by (Kemp et al., 2018). Mosquitoes are cold anesthetised and then are inoculated with 138 nL of ds306 (dsRNA targeting the cytochrome *CYP30A1*) at 5 µg/µL. A control group is made by injecting cold anesthetised mosquitoes with dsGFP.

A. **Evaluation of the *CYP306A1* inactivation in female mosquitoes**

1. *Blood fed mosquitoes 24 hours post-injection to trigger 20E synthesis (cyp306A1 expression)*
2. 24 hours after blood-meal (48 hours post injection), extract total RNA using PicoPure^{TM} RNA isolation kit (Applied Biosystems) and then treated with RNase-Free DNase (Qiagen) to remove contaminating genomic DNA.
3. Synthetize the first cDNA strand (reverse transcription) from 1 µg of each total RNA in a 20 µL reaction using Quantiscript Reverse Transcriptase (Qiagen) and oligo (dT$_{20}$) primers, following the manufacturer's instructions.
4. Check the quality and quantity of cDNA using the Nanodrop spectrophotometer. Store the cDNA samples at -20°C until further use.
5. Perform the real-time quantitative reactions (qPCR) according to the conditions described above along with the housekeeping gene *Rps7* (AGAP010592) used as normalising reference gene.

Notes: The target gene (CYP396A1) is involved in the 20-hydroxyecdysone (20E) synthesis which is triggered by blood feeding in female mosquitoes.

Data analysis

The relative quantification of *CYP306A1 expression* is determined using the $2^{-\Delta\Delta CT}$ method (Livak & Schmittgen, 2001; Schmittgen & Livak, 2008) normalized to *Rps7* as the reference gene control incorporating PCR efficiency. The mean of the threshold cycles (Ct) is normalised against the average values for the *Rps7* gene. Unpaired t-test is performed to assess significant changes in the gene expression level between the different conditions (ds306A1-injected and dsGFP-injected mosquitoes). The statistics are conducted in GraphPad Prism V.8.0.2 (San Diego, California USA) with a level o significance set at 95% confidence.

[RESULTS]

A. **qPCR Primers validation**

The designed qPCR primer sets (306A1-F1/R1, Rsp7-Fwd/Rev) were analysed for validation. The primer pair 306A1F1/R1 presents a good validatio parameters with an efficiency of 110% and R^2 value of 0.962. The primers targeting the housekeeping reference gene *Rsp7* used as reference gene for the relative gene expression analysis showed also an PCR efficiency of 103% and R^2= 0.996. In addition, the amplifications curves of each dilution sample for both *CYP306A1* and *Rsp7* genes denote the specificity of the primer pairs to selectively amplify only the corresponding target gene (**Figure 2**).

Figure 2: Amplification curves of the target genes.
A: *Rsp7* cDNA. **B:** *CYP306A1* (AGAP004665) cDNA.

A. **dsRNA synthesis and purification**

The GFP primer pair has successfully amplified the targeted GFP region with a clear amplicon (**Figure 3A**). The primers pair 306A1_T7_Fwd3/306A1_T7_Rev1 targeting the *CYP306A1* amplify one single fragment of 506 bp (**Figure 3B**). After purification both GFP and *CYP306A1* dsDNA were vissible on gel (**Figure 4A**).
The synthetised dsRNA from both *GFP* and *CYP306A1* samples were visualized on agarose gel after 1:100 dilution. Five (05) µL of the dilution were added to 1 µL of of 6X orange loading dye and mixture were run on 2% agarose gel (**Figure 4B**). After dsRNA samples digestion and purification, the purified products were diluted at 1:10 and 5 µL were electrophoresed on the gel (**Figure 4C**).

Figure 3: Gel electrophoresis showing the dsDNA after polymerase reactions using GFP and T7_primers.
A: double stranded GFP DNA (dsDNA GFP) using GFP primers. The GFP primers amplify the targeted region showing one band of approximately ~80 bp. Lanes 1 to 4 represent technical repeat reactions using the same GFP_F and GFP_R primers. MW: Molecular weight (Gene ruler 100 bp, Therm Scientific, SM0241). B: double stranded CYP306A1 DNA using T7_primers. Lane 1 to 14 represent PCR reactions using different primer pairs. Prime pairs that give one band size between 400 and 500 bp were selected. Lane 5 represents PCR product of 306A1_T7 Fwd3/306A1_T7 Rev1 primer set (506 bp).

Figure 4: Gel electrophoresis of PCR and dsRNA synthetized products.
A: GFP and 306A1 dsDNA PCR clean up products using QIAquick PCR purification kit. MW: Molecular weight (Thermo Scientific, SM0241). Lane 1 a 2: GFP samples. Lane 3 and 4: 306A1 samples. B: Electrophoresis of synthetized dsRNA after 1:100 dilution. Lane 1: synthetized Control dsRNA. Lanes 2 and 3: dsRNA from GFP samples. Lanes 4 and 5: dsRNA from CYP306A1 DNA. C: dsRNA GFP and 306A1 digested and purified samples. Lane are same as in B.

A. **Verification of the *CYP306A1* inactivation in female mosquitoes**

Quantitative PCR experiments performed on female mosquitoes injected with dsGFP and ds306A1 72h post-injection showed a significant reduction in the *CYP306A1* expression in ds306A1 injected mosquitoes compared to the dsGFP injected individuals (p= 0.0004) (**Figiure 5**).

Figure 5: Relative expression of *CYP306A1* in dsRNA injected mosquitoes 48H post blood-meal (72 hours post injection).

Recipes

3M sodium acetate (CH$_3$COONa), pH 5.2
Dissolve 12.305 g of sodium acetate in 50 mL of nuclease free water.
Adjust the pH to 5.2 with glacial acetic acid.

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Competing interests

The Authors declare that there is no conflict of interest.

References

Bhatt, S., Weiss, D., Cameron, E., Bisanzio, D., Mappin, B., & Dalrymple, U. (2015). The effect of malaria control on Plasmodium falciparum in Africa between 2000 and 2015. *Nature*, *526*(7572), 207–211.

Feyereisen, R. (1999). Insect P450 enzymes. *Annual Review of Entomology*, *44*, 507–533. https://doi.org/10.1146/annurev.ento.44.1.507

Hunt, R. H., Brooke, B. D., Pillay, C., Koekemoer, L. L., & Coetzee, M. (2005). Laboratory selection for and characteristics of pyrethroid resistance in the malaria vector Anopheles funestus. *Medical and Veterinary Entomology*, *19*(3), 271–275. https://doi.org/10.1111/j.1365-2915.2005.00574.x

Kamgang, B., Tchapga, W., Ngoagouni, C., Sangbakembi-Ngounou, C., Wondji, M., Riveron, J. M., & Wondji, C. S. (2018). Exploring insecticide resistance mechanisms in three major malaria vectors from Bangui in Central African Republic. *Pathogens and Global Health*, *112*(7), 349–359. https://doi.org/10.1080/20477724.2018.1541160

Kemp, A., Letini, ć B. D., Christian, R. N., & Koekemoer, L. L. (2018). Inoculation protocol for the African malaria vector, Anopheles arabiensis, by means of nano-injection. *African Entomology*, *26*(2), 422–428. https://doi.org/10.4001/003.026.0422

Koekemoer, L. L., Spillings, B. L., Christian, R. N., Lo, T.-C. M., Kaiser, M. L., Norton, R. A. I., Oliver, S. V., Choi, K. S., Brooke, B. D., Hunt, R. H., & Coetze M. (2010). Multiple Insecticide Resistance in *Anopheles gambiae* (Diptera: Culicidae) from Pointe Noire, Republic of the Congo. *Vector-Borne and Zoonotic Diseases*, *11*(8), 1193–1200. https://doi.org/10.1089/vbz.2010.0192

Lafont, R., Dauphin-Villemant, C., Warren, J. T., & Rees, H. H. (2017). Ecdysteroid Chemistry and Biochemistry. In *Reference Module in Life Sciences* (p. B9780128096338040000). Elsevier. https://doi.org/10.1016/B978-0-12-809633-8.04026-7

Livak, K. J., & Schmittgen, T. D. (2001). Analysis of Relative Gene Expression Data Using Real-Time Quantitative PCR and the 2−ΔΔCT Method. *Methods*, *25*(4), 402–408. https://doi.org/10.1006/meth.2001.1262

Manikandan, P., & Nagini, S. (2018). Cytochrome P450 Structure, Function and Clinical Significance: A Review. *Current Drug Targets*, *19*(1), 38–54. https://doi.org/10.2174/1389450118666170125144557

Mitchell, S. N., Kakani, E. G., South, A., Howell, P. I., Waterhouse, R. M., & Catteruccia, F. (2015). Evolution of sexual traits influencing vectorial capacity in anopheline mosquitoes. *Science (New York, N.Y.)*, *347*(6225), 985–988. https://doi.org/10.1126/science.1259435

Nair, P. C., McKinnon, R. A., & Miners, J. O. (2016). Cytochrome P450 structure–function: Insights from molecular dynamics simulations. *Drug Metabolism Reviews*, *48*(3), 434–452. https://doi.org/10.1080/03602532.2016.1178771

Ogola, E. O., Fillinger, U., Ondiba, I. M., Villinger, J., Masiga, D. K., Torto, B., & Tchouassi, D. P. (2018). Insights into malaria transmission among Anopheles funestus mosquitoes, Kenya. *Parasites & Vectors*, *11*. https://doi.org/10.1186/s13071-018-3171-3

Schmittgen, T. D., & Livak, K. J. (2008). Analyzing real-time PCR data by the comparative CT method. *Nature Protocols*, *3*(6), 1101–1108. https://doi.org/10.1038/nprot.2008.73

Scott, J. G., & Wen, Z. (2001). Cytochromes P450 of insects: The tip of the iceberg. *Pest Management Science*, *57*(10), 958–967. https://doi.org/10.1002/ps.354

Tchouakui, M., Riveron, J. M., Djonabaye, D., Tchapga, W., Irving, H., Soh Takam, P., Njiokou, F., & Wondji, C. S. (2018). Fitness Costs of the Glutathione S-Transferase Epsilon 2 (L119F-GSTe2) Mediated Metabolic Resistance to Insecticides in the Major African Malaria Vector Anopheles Funestus. *Genes*, *9*(12), 645. https://doi.org/10.3390/genes9120645

Tchouassi, D., Quakyi, I., Addison, E., Bosompem, K., Wilson, M., & Appawu, M. (2012). Characterization of malaria transmission by vector populations for improved interventions during the dry season in the Kpone-on-Sea area of coastal Ghana. *Parasit Vectors*, *5*, 212.

Warren, J. T., Yerushalmi, Y., Shimell, M. J., O’Connor, M. B., Restifo, L., & Gilbert, L. I. (2006). Discrete Pulses of Molting Hormone, 20-Hydroxyecdysone During Late Larval Development of Drosophila melanogaster: Correlations With Changes in Gene Activity. *Developmental Dynamics: An Official Publication of the American Association of Anatomists*, *235*(2), 315–326. https://doi.org/10.1002/dvdy.20626

World Health Organization. (2020). *World malaria report 2020: 20 years of global progress and challenges* (p. 299). https://www.who.int/publications-detail-redirect/9789240015791

World Health Organization. (2021). *World malaria report 2021*. World Health Organization. https://apps.who.int/iris/handle/10665/350147

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